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In vitro interactions of LytA, the major pneumococcal autolysin, with two bacteriophage lytic enzymes (Cpl-1 and Pal), cefotaxime and moxifloxacin against antibiotic-susceptible and -resistant *Streptococcus pneumoniae* strains

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***Objectives*: In an innovative therapeutic exploitation against antibiotic-resistant *Streptococcus pneumoniae*, here we have evaluated the *in vitro* activity of a purified bacterially-encoded cell wall lytic enzyme, LytA (the major pneumococcal autolysin), and compared it with those of Cpl-1 and Pal (pneumococcal phage lytic enzymes) and two antibiotics versus four pneumococcal strains.**

***Methods*: Two serotype 3, penicillin-susceptible strains and two penicillin-resistant (serotypes 19F and 19A, respectively) *S. pneumoniae* clinical isolates were used. The effect of several combinations of lytic enzymes and antibiotics (cefotaxime and moxifloxacin) was studied by chequerboard and time–kill assays, the latter at concentrations of 0.25× MIC.**

***Results*: LytA was more active than Cpl-1 and Pal. By the chequerboard technique, the combination of LytA and cefotaxime was synergistic for one of the two cefotaxime-resistant strains studied. The combined use of Cpl-1 and Pal was synergistic for three of the four strains, as was Cpl-1 with antibiotics for two of the three strains studied. In the time–kill assays, after 5 h of exposure to LytA, Cpl-1 or Pal, the mean differences in colony counts versus controls were −3.55, −2.66 and −2.71 $\log_{10}$ cfu/mL, respectively. The combination of LytA/Pal reduced the bacterial inoculum >2 log units for three of the four strains. LytA combined with cefotaxime or moxifloxacin achieved >3 log units decrease for the strains tested. Particularly, a strong synergism was observed with LytA/cefotaxime for one cefotaxime-resistant meningeal strain. LytA/moxifloxacin was synergistic for the quinolone-resistant strain when tested by time–kill methodology, and just close to synergistic (fractional inhibitory concentration index of 0.58) by the chequerboard technique. Antagonism was not observed for any combination when assayed by either method.**

***Conclusions*: LytA, Cpl-1 or Pal, alone or in combination, might prove to be effective in combination therapy, as well as in monotherapy against *S. pneumoniae*. These results suggest avenues of research to study the cell wall lytic enzymes as anti-pneumococcal therapeutic agents.**

Keywords: pneumococcus, lytic enzymes, drug resistance, killing curves, enzybiotics

Introduction

New therapeutic strategies are required for treating drug-resistant *Streptococcus pneumoniae* infections. The potential use of purified phage lytic enzymes alone, in combination or together with antibiotics is a recent line of investigation.[1–3] The pneumococcal lytic phages Cp-1 and Dp-1 encode, respectively, the lytic enzymes named Cpl-1 and Pal.[4] Purified preparations of Cpl-1 and/or Pal have been used successfully as therapeutic agents in animal models of nasopharyngeal carriage, sepsis and endocarditis

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induced by *S. pneumoniae* strains.[5–7] Furthermore *S. pneumoniae* contains four well-characterized cell wall hydrolases (LytA, LytB, LytC and Pce),[4] which could also be of interest as therapeutic agents.

In this report, we show a novel exploitation of purified LytA for the chemotherapeutic intervention of *S. pneumoniae* infections. We have evaluated whether the combined use of lytic enzymes (LytA, Cpl-1 and Pal) or an enzyme and one conventional antibiotic (cefotaxime or moxifloxacin) exhibit a synergistic effect against several *S. pneumoniae* strains.

Materials and methods

Bacterial strains, lytic enzymes and antibiotics

Four pneumococcal clinical isolates were tested. Two were penicillin-susceptible (MIC $\leq$0.01 mg/L) and belonged to serotype 3 (AR33118 and FL5629) and two were penicillin-resistant (MIC 4 and 8 mg/L), one of serotype 19F (MJD3693) and the other of serotype 19A (South African strain 8249). LytA, Cpl-1 and Pal were purified by affinity chromatography in DEAE-cellulose as previously described for other choline-binding enzymes.[8] Cefotaxime was purchased from Sigma Chemicals Co. (St Louis, MO, USA) and moxifloxacin (Bayer AG, Leverkusen, Germany) was kindly provided by the manufacturer.

MIC determination

The MICs of LytA, Cpl-1, Pal, cefotaxime and moxifloxacin were determined by broth microdilution following the CLSI methodology.[9]

Chequerboard broth microdilution method for synergy testing

Interactions between two enzymes and between one enzyme and one antibiotic were tested by the chequerboard method[10] in microtitre plates in the same broth as used for MIC determinations. All compounds were tested alone and in combination at seven concentrations that usually ranged from one-sixteenth to four times the MIC. Inocula were prepared at a bacterial concentration of $\sim 5 \times 10^4$ cfu per well. The final volume was 100 μL per well. Trays were aerobically incubated at 35°C for ~22 h. The results were interpreted by the pattern they showed on an isobologram and the fractional inhibitory concentration (FIC) for each compound was calculated. The FIC index (FICI) was determined by summing the separate FICs for each of the compounds present in that well. Interactions between any enzyme and cefotaxime or moxifloxacin were not assessed against organisms fully susceptible to these antibiotics. Each test was performed at three separate times and the FICI values were expressed as means. Synergy was defined by an FICI $\leq$0.5, antagonism by an FICI $>$4.0 and no interaction by an FICI $>$0.5–4.

Time–kill determinations

Selected combinations were studied using time–kill kinetic analysis; particularly for the four clinical isolates for which the LytA-containing combinations achieved FICI $<$0.8. The assays were performed in broth with the same composition as used for MIC determinations with each compound alone or in combination at 0.25× MIC. Bacteria were grown in broth to mid-log phase at a final titre of $\sim 10^7$ cfu/mL. Inoculated tubes were incubated at 35°C. Samples (100 μL) were removed after 1, 3 and 5 h and plated on blood agar plates for titre determination. Each assay was performed in duplicate. Synergy was defined as a $\geq$2 $\log_{10}$ decrease in cfu/mL between the combination and its most active agent, and the number of viable organisms in the presence of the combination was $\geq$2 $\log_{10}$ cfu/mL below the starting inoculum.

Results

The MIC values (LytA, Cpl-1, Pal, cefotaxime and moxifloxacin) were: strain MJD3693 (16, 32, 256, 4 and 0.25 mg/L), strain AR33118 (8, 16, 32, $\leq$0.01 and 0.12 mg/L), strain 8249 (2, 8, 128, 4 and 0.25 mg/L) and strain FL5629 (4, 16, 32, $\leq$0.01 and 8 mg/L).

The interactions of LytA, Cpl-1, Pal, cefotaxime and moxifloxacin, as determined by the chequerboard technique, are shown in Table 1. The combination of LytA with Cpl-1 was indifferent against the four strains, although for strain FL5629 an FICI of 0.67 was achieved. Synergy between LytA and Pal was not found for the four strains, but the FICIs were lower than 0.8. The combination of LytA with cefotaxime was synergistic for strain MJD3693, one cefotaxime-resistant strain (FICI = 0.18), whereas it was not for the second cefotaxime-resistant isolate, strain 8249 (FICI = 0.71). For the moxifloxacin-resistant strain (FL5629), synergy between LytA and moxifloxacin was not observed (FICI = 0.58).

Synergy was also found with the following combinations: Cpl-1 with Pal for three out of the four strains tested; Cpl-1 with cefotaxime for one of the two cefotaxime-resistant strains tested (strain MJD3693) and Cpl-1 with moxifloxacin for the quinolone-resistant isolate (strain FL5629). The combination of Pal with either cefotaxime or moxifloxacin did not prove to be synergistic against the antibiotic-resistant strains. Antagonism was not observed for any combination, against any of the strains tested.

Figure 1 shows the results of the time–kill experiments for LytA, Pal, Cpl-1, cefotaxime and moxifloxacin at a fixed concentration of 0.25× MIC, alone and in combination.

Table 1. FICIs for combinations against four pneumococcal strains

Compound	Combined with	Strain			
		MJD3693	AR33118	8249	FL5629
LytA	Cpl-1	1.27	1.06	0.91	*0.67*
	Pal	*0.54*	*0.79*	*0.57*	*0.55*
	cefotaxime	***0.18***	ND	*0.71*	ND
	moxifloxacin	ND	ND	ND	*0.58*
Cpl-1	Pal	**0.50**	**0.42**	**0.34**	0.60
	cefotaxime	**0.41**	ND	0.54	ND
	moxifloxacin	ND	ND	ND	**0.42**
Pal	cefotaxime	0.62	ND	0.56	ND
	moxifloxacin	ND	ND	ND	1.05

Synergistic combinations are in bold; combinations that were also studied by time–kill method are in italics.
ND, not determined (as the organisms were fully susceptible to the antibiotics).

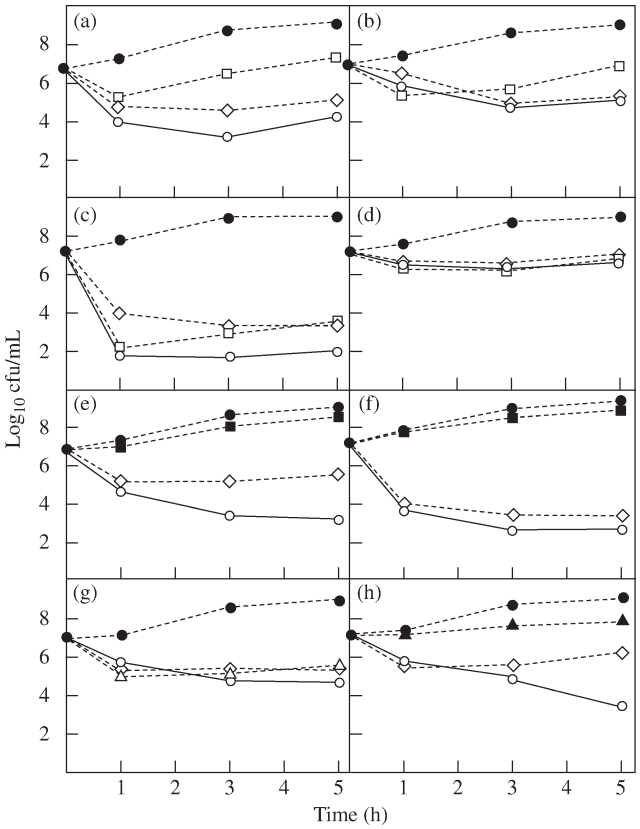

Figure 1. Time–kill assays with five compounds each at 0.25× MIC alone and in combination (solid lines) against four pneumococcal strains. (a–d) Strains MJD3693, AR33118, 8249 and FL5629, respectively. LytA, open diamonds; Pal, open squares; combination, open circles. (e and f) Strains MJD3693 and 8249, respectively. LytA, open diamonds; cefotaxime, filled squares; combination, open circles. (g) Strain FL5629. LytA, open diamonds; Cpl-1, open triangles; combination, open circles. (h) Strain FL5629. LytA, open diamonds; moxifloxacin, filled triangles; combination, open circles. All control values are represented by filled circles.

No combination was antagonistic by the time–kill assays. After 1, 3 and 5 h of single exposure to LytA and Pal, the mean differences in colony counts for the four strains tested versus controls were −2.24, −3.47 and −3.55, and −2.89, −3.19 and −2.71 $\log_{10}$ cfu/mL. On the other hand, Cpl-1 alone showed differences in bacterial titres versus controls of −1.64, −2.71 and −2.66 $\log_{10}$ cfu/mL for the strain tested. In contrast, at all time points, the differences in colony counts achieved by cefotaxime or moxifloxacin alone were lower than −0.77 $\log_{10}$ cfu/mL versus controls for the strains tested. Bactericidal activity (reduction of ≥3 $\log_{10}$ cfu/mL from the initial inoculum) was mostly achieved with LytA and Pal alone for one of the four strains (strain 8249). Furthermore, LytA/Pal was also bactericidal for two out of the four strains (strains MJD3693 and 8249). LytA/cefotaxime also showed bactericidal activity for the two strains studied (strains MJD3693 and 8249), as did LytA/moxifloxacin for the single strain studied (strain FL5629).

Synergistic effects were achieved after 5 h of exposure to LytA/cefotaxime against strain MJD3693 (2.48 $\log_{10}$ decrease in cfu/mL between the combination and LytA) and to LytA/moxifloxacin against strain FL5629 (2.42 $\log_{10}$ decrease in cfu/mL between the combination and LytA). For these two strains, these enzyme/antibiotic combinations showed FICIs of 0.18 and 0.58, respectively. Therefore, only in the first case, the FICI supported a synergistic effect when applying stringent criteria. Although no synergism was found with LytA combined with cefotaxime in the other cefotaxime-resistant strain (8249), this strain was highly susceptible to LytA alone (more than 4 $\log_{10}$ reduction) making it difficult to demonstrate such as effect.

Discussion

The results of the present study confirm the superior *in vitro* activity of LytA compared with those of Cpl-1 and Pal in terms of a lower MIC value for several *S. pneumoniae* strains. In contrast to the antibiotic activity, LytA and the other two enzymes reduced the bacterial inoculum very rapidly at concentrations of 0.25× MIC. Furthermore, one cefotaxime-resistant (MIC 4 mg/L) strain (8249) had a LytA MIC of 2 mg/L, being rapidly bactericidal at concentrations of 0.25× MIC, and such activity was maintained up to 5 h. Our results show that LytA displayed a potent synergistic effect with cefotaxime against one of the two cefotaxime-resistant strains by the two techniques used (MJD3693). Furthermore, LytA combined with moxifloxacin was also synergistic against the moxifloxacin-resistant strain tested (FL5629), but only by time–kill assays although the FICI (0.58) of such a combination was close to 0.5. For the second cefotaxime-resistant strain (8249), it was not possible to demonstrate synergism with LytA/cefotaxime, probably due to the strong bactericidal activity of LytA alone. By the chequerboard technique, no synergism between Cpl-1 and LytA was observed for any strain. The combination of Cpl-1 plus Pal resulted in synergism against three out of the four pneumococcal strains tested. Moreover, this synergistic effect has been confirmed with a sepsis model:[5] the combination of Cpl-1 and Pal was more effective than either agent alone in prolonging survival in experimental penicillin-resistant pneumococcal sepsis in mice. In our experiments, the combination of Cpl-1 and one antibiotic, cefotaxime or moxifloxacin, indicated synergism, respectively, for one cefotaxime-resistant strain (MJD3693; FICI = 0.41) and for the moxifloxacin-resistant strain (FL5629; FICI = 0.42). This latter finding was in contrast to a previous report, in which Cpl-1 showed no interaction when combined with levofloxacin.[3] Noticeably, the synergistic effect of Cpl-1 combined with cefotaxime was shown against the same cefotaxime-resistant isolate for which LytA combined with cefotaxime was synergistic, but the combination of Cpl-1/cefotaxime against the other cefotaxime-resistant strain (8249) achieved an FICI of 0.54, close to a synergistic value. On the other hand, the combination of Pal and cefotaxime demonstrated no interaction for the two pneumococci tested.

The combinations of LytA giving FICIs <0.8 were confirmed with the more dynamic interaction provided by the time–kill kinetic study system using one-fourth times the MIC of each agent alone or combined. The results of time–kill kinetic curves confirmed the chequerboard data in that none of the combinations resulted in antagonism and seven out of the eight time–kill tests showed the same results as those obtained by the chequerboard technique (synergism with LytA/cefotaxime against the meningeal cefotaxime-resistant strain, and indifference with

the rest of the combinations). The unique discrepancy was shown for the combination of LytA/moxifloxacin that was synergistic by time–kill testing and indifferent by the chequerboard procedure, but having an FICI of 0.58.

We provide the first evidence that the exogenous addition of purified LytA may well be considered a good candidate for a new generation of antibacterial agents to treat antibiotic-resistant *S. pneumoniae* infections. In an era of global spreading of antibiotic-resistant pneumococci, the potential role of cell wall hydrolases, such as LytA, Cpl-1 and Pal, as therapeutic agents warrants further investigation.

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Transparency declarations

None to declare.

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